Exclusionary Drug Screen by UPLC-ESI-FTMS

1 Introduction

A rapid ultra-performance liquid chromatography electrospray Fourier transform mass spectrometry (UPLC-ESI-FTMS) method can be used to quickly screen blood and urine specimens for common drugs of abuse and prescription medications. The amount of a detected analyte can be estimated by comparing the response (peak area) for the analyte to that for a corresponding internal standard. Positive findings are confirmed via a second technique.

2 Scope

This procedure allows for the screening of blood and urine specimens for the presence of cocaine and metabolites, opioids, antihistamines, benzodiazepines and other hypnotics (see Section 13 for a list of target analytes and limits of detection). This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Urine specimens are subjected to enzymatic hydrolysis. Blood and hydrolyzed urine specimens are diluted with buffer and made alkaline before purification via supported liquid extraction. Final extracts are analyzed by UPLC-ESI-FTMS in full scan mode.

4 Specimens

0.3 mL of blood or urine is required for this assay.

5 Equipment/Materials/Reagents

a. 13 x 100 mm and 16 x 100 mm test tubes

b. Ammonium Acetate (99.999% purity)

c. Deionized Water

d. Acetic Acid, glacial (17 M, ACS grade)

e. pH meter

f. Ammonium Acetate Buffer (0.5 M, pH5):
Add 3.854 g ammonium acetate to a 100-mL volumetric flask containing about 75 mL deionized water. Mix well to dissolve. Add glacial acetic acid until pH registers between 4.5 and 5.5. Bring to volume with deionized water and mix well. Store refrigerated in glass or plastic. Stable at least 3 months.

g. Vortex mixer

h. Ammonium hydroxide, concentrated (15 M, ACS grade)

i. Ammonium Hydroxide (4.5 M):
Mix 1.4 mL deionized water and 0.6 mL concentrated ammonium hydroxide in a test tube. Prepare fresh daily.

j. β-glucuronidase (>120,000 u/mL β glucuronidase activity; from Red Abalone, *H. Rufescena*; available from Kura Biotec)

k. Supported Liquid Extraction (SLE) cartridges (Biotage Isolute SLE+; 2 mL sorbent mass; part number 820-0290-D)

l. Dichloromethane (Optima grade)

m. Isopropanol (HPLC grade)

n. Elution Solvent (95/5 Dichloromethane/Isopropanol):
Combine 95 mL dichloromethane and 5 mL isopropanol and mix well. Store at room temperature in brown glass. Stable for at least one month.

o. Vacuum extraction box

p. Heating block

q. Heated evaporator

r. Acetonitrile (Optima grade)

s. Water/Acetonitrile (95/5):
Combine 9.5 mL deionized water and 0.5 mL acetonitrile and mix well. Store at room temperature in glass. Prepare fresh weekly.

t. 0.2 µm centrifuge tubes

u. Autosampler vials with caps

v. Waters Cortecs® C18, 1.6 µ; 2.1 x 50 mm UPLC Column with a Cortecs® C18 1.6 µ

precolumn

w. Seal Wash Solvent and Weak Wash Solvent (Water/Acetonitrile; 90/10):
Combine 450 mL deionized water and 50 mL acetonitrile and mix well. Store at room temperature in glass. Stable for at least two weeks.

x. Methanol (Optima grade)

y. Strong Wash Solvent (Methanol/Acetonitrile/Water/Isopropanol; 45/40/10/5):
Combine 90 mL methanol, 80 mL acetonitrile, 20 mL deionized water and 10 mL isopropanol and mix well. Store at room temperature in glass. Stable for at least six months.

z. Liquid chromatograph capable of ultra-performance liquid chromatography coupled to a mass spectrometer capable of 70,000 resolution.

aa. LC Mobile Phase B (Acetonitrile with 0.1% Formic Acid): Combine 500 mL acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least one month.

bb. LC Mobile Phase A (5 mM Ammonium Formate with 0.1% formic acid): Add 0.158 g ammonium formate to a 500 mL volumetric flask. Add approximately 400 mL deionized water and mix well. Add 0.5 mL formic acid, and QS with deionized water. Store in glass at room temperature. Stable for one week.

6 Standards and Controls

a. Negative Control Blood:
Purchased from Cliniqa or another suitable commercial source. Stability and storage determined by manufacturer. A Negative Control Blood will be analyzed in every blood batch.

b. Negative Control Urine:
Obtained in-house. Store refrigerated or obtain fresh. Stable at least two years. Alternatively, synthetic urine may be purchased from Dynatek. Storage and stability determined by the manufacturer. A Negative Control Urine will be analyzed in every urine batch.

c. Internal Standard Components:
Purchased from Cerilliant, International or another suitable vendor as 0.1 mg/mL solutions. Storage and stability determined by manufacturer.
- benzoylecgonine-d_3 or benzoylecgonine-d_8
- morphine-d_3
- hydrocodone-d_3

- oxycodone-d_6
- clonazepam-d_4
- 7-aminoclonazepam-d_4
- α-hydroxyalprazolam-d_5
- alprazolam-d_5
- oxazepam-d_5
- diazepam-d_5
- zolpidem-d_6
- diphenhydramine-d_3

d. Internal Standard Working Solution:
Combine the listed volumes of each Internal Standard Component in a 100-mL volumetric flask and bring to the mark with acetonitrile. Store refrigerated in glass. Stable for at least two years.

Component	Volume (µL)	Final Concentration (ng/mL)	Final Concentration in Specimen (ng/mL)
benzoylecgonine-d_3 or benzoylecgonine-d_8	120	120	10
morphine-d_3	120	120	10
hydrocodone-d_3	60	60	5
oxycodone-d_6	60	60	5
clonazepam-d_4	60	60	5
7-aminoclonazepam-d_4	36	36	3
α-hydroxyalprazolam-d_5	36	36	3
alprazolam-d_5	36	36	3
oxazepam-d_5	36	36	3
diazepam-d_5	36	36	3
zolpidem-d_6	24	24	2
diphenhydramine-d_3	36	36	3

e. Positive Control Components
Purchased from Cerilliant, International or another suitable vendor as 1.0 mg/mL solutions. Storage and stability determined by manufacturer.

- Benzoylecgonine
- Diazepam
- Diphenhydramine
- Oxycodone
- Morphine-3-β-glucuronide (or morphine-6-β-glucuronide)

f. Positive Control Stock Solution (5.0 µg/mL):
Combine 82 µL of the morphine glucuronide solution and 50 µL of the remaining

component solutions in a 10-mL volumetric flask and bring to the mark with acetonitrile. Store in glass at <0ºC. Stable for at least two years.

g. Positive Control Working Solution (0.15 µg/mL):
Add 300 µL of the Positive Control Stock Solution to a 10-mL volumetric flask and bring to the mark with 1:1 methanol:water. Store in glass at <0ºC. Stable for 6 months.

h. Positive Control (10 ng/mL):
Add 20 µL of the Positive Control Working Solution (0.15 µg/mL) to 0.3 mL Negative Control (urine or blood, as needed) on the day of analysis. Urine Positive Controls will be hydrolyzed enzymatically along with the Negative Control and case samples.

A Positive Control will be analyzed with every batch.

i. Performance Standard Mix:
Mix 0.025 mL of the Internal Standard Working Solution with 0.375 mL of deionized water. Prepare fresh on day of use.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

8.1 Blood specimen preparation:

a. Add 0.3 mL of each sample and control to a properly labeled 13 x 100 mm test tube.

b. Add 25 µL of the Internal Standard Working Solution to each sample.

c. Add 0.7 mL Ammonium Acetate Buffer (0.5 M, pH5).

d. Add 0.6 mL Deionized Water and vortex.

e. Add 80 µL Ammonium Hydroxide (4.5 M) and vortex.

8.2 Urine specimen preparation:

a. Add 0.3 mL of each sample and control to a properly labeled 13 x 100 mm test tube.

b. Add 25 µL of the Internal Standard Working Solution to each sample.

c. Enzymatic Hydrolysis:

i. Add 0.6 mL Ammonium Acetate Buffer (0.5 M, pH5).

ii. Add 100 µL β-glucuronidase (H. Rufescena).

iii. Vortex, cap and incubate for 30 minutes at approximately 68°C.

iv. Cool to approximately room temperature.

v. Add 0.6 mL Deionized Water and vortex.

d. Add 80 µL Ammonium Hydroxide (4.5 M) and vortex.

8.3 Extraction (for all specimens):

a. Load samples onto SLE cartridges by gravity. (A brief application of vacuum will be necessary to start loading.)

b. Allow to stand for 5 minutes.

c. Apply 3 mL of Elution Solvent (95/5 Dichloromethane/Isopropanol) and allow to absorb.

d. Allow to stand for 5 minutes. Do not apply vacuum.

e. Elute by gravity into 16 x 100 mm test tubes with 2 x 4 mL Elution Solvent (95/5 Dichloromethane/Isopropanol). Briefly apply full vacuum to complete elution.

f. Evaporate at approximately 45°C. When volume reaches 0.5 – 1 mL, briefly vortex before evaporating to dryness.

g. Reconstitute with 0.1 mL Water/Acetonitrile (95/5).

h. Filter with 0.2 µm centrifuge tubes.

i. Transfer each sample to properly labeled autosampler vials.

j. Analyze 20 µL by UPLC-ESI-FTMS.

9 Instrumental Parameters

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters				Column Parameters	
A: 5 mM ammonium formate in 0.1% formic acid	total flow	0.50 mL/min			type	Waters Cortecs™ C18
	time (min)	% A	% B	Curve	length	50 mm
B: 0.1% formic acid in acetonitrile	0	95	5	Initial	internal diameter	2.1 mm
	0.1	95	5	9	particle size	1.6 µ
Event table: at 6.25 min, cycle inject valve	0.9	95	5	9	temperature	30°C
	2.67	60	40	6	5 x 2.1 mm precolumn Autosampler Temp: 14°C Weak Wash Vol: 1000 µL Strong Wash Vol: 800 µL Sample Loop: 20 µL Needle Overfill Flush: 5 µL	
	4.67	60	40	6		
	5.56	0	100	6		
	7.0	0	100	6		
	7.25	95	5	6		
	10	95	5	6		
	total time	10 min				

9.2 Mass Spectrometer Parameters

Parameter	Value
Method duration	10 min
Mode	ESI, Full scan MS
Polarity	positive
Microscans	1
Resolution	70,000
AGC Target	1e6
Maximum IT	50 ms
Scan Ranges	1
Scan Range	180-525 m/z
Spectrum data type	profile

10 Decision Criteria

10.1 Performance Standard Decision Criteria

In addition to the performance checks specified in the instrument standard operating procedure, a performance standard mix is analyzed through the analytical column to monitor the performance of the column.

10.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each analyte in the performance standard should have reasonable peak shape.

The retention times of the 12 analytes should be within ±0.05 minutes of the previous run of the performance standard. If the retention times have shifted more than 0.05 minutes, the column or precolumn may need to be changed, or the column may not be at equilibrium.

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

10.1.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the 12 analytes in the performance standard should be present. The following molecular ions should be present as the base peak for each analyte, with a tolerance of ±5 mmu:

Internal Standard	Mass
d_5-alprazolam	314.12150
d_5-hydroxyalprazolam	330.11650
d_4-clonazepam	320.07350
d_4-7-aminoclonazepam	290.09927
d_5-diazepam	290.11030
d_5-oxazepam	292.08957
d_6-zolpidem	314.21340
d_6-oxycodone	322.19200
d_3-morphine	289.16260
d_3-hydrocodone	303.17830
d_3-benzoylecgonine or d_8-benzoylecgonine	293.1577 or 298.1889
d_3-diphenhydramine	259.1887

10.2 Unknown Sample Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

10.2.1 Batch Acceptance

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the 12 internal standards should be detected in each Control. If any of the internal standards are not detected in the Control, data for the drug class that the internal standard falls into should be interpreted with care for the batch. The analytes in the Positive Control should be detected; for hydrolyzed urine batches morphine should be detected. Positive control failures will be evaluated and reanalysis may be necessary.

10.2.2 Unknown Sample Acceptance

All 12 internal standards should be detectable in the unknown sample. If any of the internal standards are not detected in a case sample, the data for that case should be interpreted with care.

10.2.3 Unknown Sample Compound Detection

This procedure is used for screening purposes only and not for the identification of specific drugs.

10.2.3.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution.

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

Unknown sample files are reviewed using M+1 layouts with a mass tolerance of ±2.5 mmu. Any peak within ±0.05 minutes of the target retention time should be explored as possible positive results.

10.2.3.2 Mass Spectrometry

Following is a list of validated analytes for this method and their corresponding molecular ions and retention times (Table 1). Detection of a peak at the proper retention time is an indication that the analyte may be present, and confirmation testing will be performed.

Table 1: Analytes with retention times and molecular ions

Analyte	Retention Time (RT; min) / RT of labeled Internal Standard, if applicable	Molecular Ion (M+1)
Benzodiazepines and Metabolites		
α-hydroxyalprazolam	2.9 / 2.9	325.0851
α-hydroxymidazolam	2.67	342.0804
α-hydroxytriazolam	2.9	359.0461
7-aminoclonazepam	2.07 / 2.07	286.0742
7-aminoflunitrazepam	2.27	284.1194
alprazolam	3.06 / 3.06	309.0902
bromazepam	2.66	316.0080
chlordiazepoxide	2.46	300.0898
clonazepam	3.07 / 3.07	316.0484
desalkylflurazepam	3.19	289.0539
desmethylflunitrazepam	2.95	300.0779
diazepam	3.7 / 3.67	285.0789
estazolam	3	295.0745
etizolam	3.25	343.0778
flunitrazepam	3.22	314.0936
flurazepam	2.71	388.1586
lorazepam	3.04	321.0192
lormetazepam	3.43	335.0348
medazepam	2.71	271.0996
midazolam	2.67	326.0855
nordiazepam	3.19	271.0633
oxazepam	2.98 / 2.98	287.0582
phenazepam	3.45	348.9738
prazepam	5.33	325.1102
temazepam	3.27	301.0738
tetrazepam	3.68	289.1102
triazolam	3.12	343.0511
Opioids and Metabolites		
6-acetylmorphine	1.77	328.1543
codeine	1.54	300.1594
dihydrocodeine	1.5	302.1751
dihydromorphone	0.49	288.1594

Analyte	Retention Time (RT; min) / RT of labeled Internal Standard, if applicable	Molecular Ion (M+1)
EDDP	2.87	278.1903
hydrocodone	1.8 / 1.79	300.1594
hydromorphone	0.86	286.1438
morphine	0.53 / 0.53	286.1438
norcodeine	1.46	286.1438
normorphine	0.44	272.1281
noroxycodone	1.69	302.1387
oxycodone	1.72 / 1.72	316.1543
oxymorphone	0.64	302.1387
Cocaine and Metabolites		
ecgonine methyl ester	0.26	200.1280
benzoylecgonine	1.96 / 1.94	290.1387
cocaethylene	2.5	318.1700
cocaine	2.3	304.1543
Antihistamines and Related Compounds		
brompheniramine	2.5	319.0804
chlorpheniramine	2.43	275.1309
dextromethorphan	2.65	272.2008
dextrorphan	2.11	258.1852
diphenhydramine	2.73 / 2.71	256.1695
doxylamine	1.94	271.1804
hydroxyzine	2.98	375.1833
norchlorcyclizine	2.98	287.1309
pheniramine	1.93	241.1699
tetrahydrozoline	1.87	201.1386
Hypnotics		
zaleplon	2.86	306.1349
zolpidem	2.35 / 2.35	308.1757
zopiclone	2.13	389.1123
Antidepressant		
duloxetine	3.02	298.1260

11 Calculations

Not applicable.

12 Measurement Uncertainty

Not applicable.

13 Limitations

a. Limit of Detection:

Table 2: Limits of Detection

Analyte	Blood LOD (ng/mL)	Urine LOD (ng/mL)
Benzodiazepines and Metabolites		
α-hydroxyalprazolam	1	1
α-hydroxymidazolam	1	1
α-hydroxytriazolam	1	1
7-aminoclonazepam	1	1
7-aminoflunitrazepam	1	1
alprazolam	1	1
bromazepam	1	1
chlordiazepoxide	1	1
clonazepam	1	1
desalkylflurazepam	1	1
desmethylflunitrazepam	1	1
diazepam	1	1
estazolam	1	1
etizolam	1	1
flunitrazepam	1	1
flurazepam	1	1
lorazepam	1	1
lormetazepam	1	1
medazepam	5	1
midazolam	1	1
nordiazepam	1	1
oxazepam	1	1
phenazepam	1	1
prazepam	5	1

Analyte	Blood LOD (ng/mL)	Urine LOD (ng/mL)
temazepam	1	1
tetrazepam	1	1
triazolam	1	1
Opioids and Metabolites		
6-acetylmorphine	1	3
codeine	1	1
dihydrocodeine	1	1
dihydromorphone	1	1
EDDP	1	1
hydrocodone	1	1
hydromorphone	1	3
morphine	1	3
norcodeine	3	3
normorphine	1	1
noroxycodone	1	1
oxycodone	1	1
oxymorphone	1	1
Cocaine and Metabolites		
ecgonine methyl ester	1	5
benzoylecgonine	1	1
cocaethylene	1	1
cocaine	1	1
Antihistamines and Related Compounds		
brompheniramine	1	1
chlorpheniramine	1	1
dextromethorphan	10	1
dextrorphan	5	1
diphenhydramine	5	1
doxylamine	5	1
hydroxyzine	50	1
norchlorcyclizine	5	1
pheniramine	1	1
tetrahydrozoline	1	1
Hypnotics		
zaleplon	1	1

Analyte	Blood LOD (ng/mL)	Urine LOD (ng/mL)
zolpidem	3	3
zopiclone	1	1
Antidepressant		
duloxetine	50	5

b. Specificity: No known interferences. However, this procedure will be used as a screen only and all positive findings will be confirmed by a second procedure.

c. This procedure is not suitable to screen specimens for buprenorphine, norbuprenorphine or the carboxylic metabolite of zolpidem.

15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

16 References

FBI Laboratory Safety Manual

Rapid Screening for Drugs of Abuse in Biological Fluids by Ultra High Performance Liquid Chromatography/Orbitrap Mass Spectrometry. Jagerdeo E, et al. Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences. 2016 Aug 1; 1027:11-8.

Rev. #	Issue Date	History
0	08/20/15	New document
1	04/29/16	Section 2, Table 1 and Table 2 were updated to add additional analytes and analyte classes. Section 5.v. was updated to add details on the precolumn. Section 5 bb. was updated to reflect lower number of significant figures on mass of buffer salt. Tolerances were tightened in sections 10.1.1, 10.1.2 and 10.2.3.1. Clarified acceptance criteria in 10.2.1 and 10.2.2. Added a note in 14.c concerning drugs and metabolites not suitable to screen for with this method.
2	08/25/16	In 5.aa, 5.bb, 9.1, and Appendix 2, switched Mobile Phase A to aqueous and Mobile Phase B to organic. In 5.bb, removed phrase "at least". For internal standards, removed d_3-cocaine, added d_3-diphenhydramine, and allowed for use of d_3 or d_8-benzoylecgonine which caused updates to 6.c, 6.d and 10.1.2. In 6.d, added final concentration of internal standard in specimens. Updated wording in 10.2.3. Added internal standard retention times to Table 1. Added lot number slots for washes to instrument sheet in Appendix 2.
3	02/09/18	Rewrote Sections 6 (e-i), adding a Positive Control that adds four analytes (in addition to the existing morphine glucuronide). Changed Section 5t and 8.3h to read 0.2 micron filters instead of 0.45 micron filters. Updated scope language in Section 2. Specified that Negative Controls will be run in batches, updating Sections 6a and 6b. Updated 8.2c(iii and iv) to "approximately"; also renumbered this section as previous version used nested alphabetical scheme. Added "approximately" to 8.3f. Updated 10.2.1 to account for inclusion of Positive Controls. Added a published reference to Section 16. Updated approvers. Updated Section 1 "the" to "a corresponding internal standard".

Approval

Redacted - Signatures on File

Toxicology Technical Lead: Date: 02/08/2018

Chemistry Unit Chief: Date: 02/08/2018

QA Approval

Quality Manager: Date: 02/08/2018

Appendix 1: Abbreviated version of the Procedure for bench use

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Appendix 2: Abbreviated version of the Instrumental Conditions for bench use

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